Neurochem Res (2010) 35:2124–2134
DOI 10.1007/s11064-010-0325-x

ORIGINAL PAPER

Astrocytes Express *N*-Methyl-D-Aspartate Receptor Subunits in Development, Ischemia and Post-Ischemia

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Accepted: 11 November 2010 / Published online: 30 November 2010

Abstract The expression of the *N*-methyl-D-aspartate receptor (NMDA-R) in astrocytes is controversial. The receptor is commonly considered neuron-specific. We showed that astrocytes in primary cultures differentially expressed mRNA of NMDA-R subunits, NR1, NR2A and NR2B, in development, ischemia and post-ischemia. One-week-old cultures expressed detectable NR1 mRNA, which fell significantly at 2 weeks and became barely detectable at 4 weeks. NR2A and NR2B mRNA were both significantly up-regulated from 1 to 2 weeks. In 4 weeks, 2 h of ischemia caused a significant up-regulation of NR1 and NR2B mRNA; while 6 h caused down-regulation of NR2A mRNA. Under 3 h of post-ischemia, only NR1 mRNA was increased. Ischemia induced the expression of major NMDA-R effecter, nitric oxide synthase 1, which was unaffected by AMPA-R antagonist CNQX, but dose-dependently inhibited by NMDA-R specific antagonist MK-801. These findings reflected that astrocyte could express inducible functional NMDA receptors without the presence of neurons.

Keywords NOS1 · MK-801 · NR1 · NR2

Introduction

N-methyl-D-aspartate receptor (NMDA-R), an ionotropic glutamate receptor, plays an important role in both physiological and pathological conditions [1, 2]. It has been heavily studied in neurons both in vivo and in cultures for a variety of significant neural processes, such as long term

Special Issue: In Honor of Dr. Abel Lajtha.

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potentiation, learning, memory, pain and injury [3–5]. Activation of NMDA-R triggers nitric oxide synthase 1 (NOS1) cascades to increase the NO level, leading to activation of soluble guanylate cyclase and production of cyclic GMP in the brain [6–8]. This NMDA-R/NOS pathway is involved in a variety of cellular events, such as synaptic plasticity, neurotransmitter release and neurotoxicity [9, 10].

Astrocytes are responsible for maintaining environmental homeostasis in the brain, especially by controlling extracellular glutamate levels [11–14]. Though they had been shown to express many channels and receptors except NMDA-R [15–19], accumulative evidences have suggested that astrocytes might actually express functional NMDA-R [20–22]. In one study, Krebs et al. reported the expression of functional NR2B, an NMDA-R subunit, in astrocytes in co-culture with neurons after anoxia treatment; they believed that this expression might be induced in astrocytes by the neuronal components [23]. Regardless, the presence of NMDA-R in astrocytes indicates a more extensive ion control potential for the cells and greatly expands the list of possible astrocytic functions in the brain. Thus, it is interesting to clarify whether astrocytes are capable of independently expressing NMDA-R.

In this study, the mRNA levels of three major NMDA-R subunits, NR1, NR2A and NR2B, were studied in primary cultures of astrocytes during development, ischemia and post-ischemia. The existence of functional NMDA-R was confirmed in astrocytes by measuring NOS1 protein levels in these cells under ischemia and post-ischemia treatments.

Experimental Procedure

Primary Cultures of Astrocytes and Neurons

Primary cultures of cortical astrocytes were prepared from newborn ICR mice as described previously [24, 25]. Cerebral cortices with meninges removed were cut into small cubes (<1 mm^3) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Life Technologies, Inc., NY, USA). Dissociated cell suspension was sequentially passed through 70 and 10 μm sterile nylon filters (Spectra/Mesh®, Spectrum Medical Industries, Inc., TX, USA). Fetal bovine serum (FBS; HyClone Laboratories Inc., Australia) was added to 10% of the final volume. The mixture was plated in 35 mm Falcon tissue culture dishes at 4×10^5 cell/ml (Becton–Dickinson & Co., CA, USA). Cultures were fed twice weekly with DMEM (10% FBS) for the first 2 weeks and DMEM (7% FBS) for the following 2 weeks.

Primary cultures of cortical neurons were adopted as a positive control for NMDA-R mRNA expression studies. Neurons were prepared from embryonic ICR mice as previously described [26]. Cytosine arabinoside (40 μM) (Sigma–Aldrich, MO, USA) was added on day 3 to inhibit the growth of the astrocytes. Cultured neurons were used on day 7 [27].

Immunofluorescent Staining

Antibodies against glial fibrillary acidic protein (GFAP) (1:100), microtubule-associated protein 2 (MAP2) (1:100) and NR2B (1:25) were from Santa Cruz Biotechnology (CA, USA). Antibody against NR1 (1:50) was from Upstate/Millipore (MA, USA). Primary cultures were fixed on ice in 4% paraformaldehyde and permeablized by 0.3% Triton X-100 for 5 min. After blocking with 3% bovine serum albumin in phosphate buffered saline (PBS), the cultures were incubated with primary antibody in 4°C overnight. FITC or TRITC conjugated secondary antibody (1:200) from Beijing Zhongshan Biotechnology Co. (China) was used.

Measurements of NMDA-R Subunits mRNA

NR1, NR2A and NR2B are the most widely investigated for NMDA-R, therefore we focused to investigate on these three NMDA-R subunits in this study.

Total RNA was prepared from cultures by TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RT-PCR was carried out to synthesize cDNA from 1 μg total RNA [26, 28]. The primer sequences of NMDA-R subunits NR1, NR2A and NR2B were adapted from Morley et al. [29]. Actin (forward: 5′-cagccttccttcttgggtat-3′; reverse: 5′-gctcagtaacagtccgccta-3′) was designed as an internal control. However, we found that the actin interfered with NR2B primers in the PCR reactions, and therefore tubulin was used (forward: 5′-attggcaaggagatcattgac-3′; reverse: 5′-caacctcctcataatccttct-3′) as the internal control for NR2B semi-quantifications.

Glutamate and Hydrogen Peroxide Treatments

Astrocytes were treated with 1 mM glutamate (Sigma–Aldrich, MO, USA) or 200 μM hydrogen peroxide (H_2O_2) (Beijing Chemical Works, China) for 0, 4 and 8 h. In the glutamate transporter inhibitor treatment, 50 μM L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-*trans*-PDC) (Sigma–Aldrich, MO, USA) was added to the cultures 30 min before glutamate treatment. Total RNA from these cultures was extracted and subjected to RT-PCR analysis for the three NMDA-R subunits.

Ischemia and Post-Ischemia Treatments

The medium for the ischemia experiment was glucose and serum-free DMEM, and was equilibrated overnight in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2, 37°C; Forma Scientific Inc., OH, USA). We have previously reported the use of this model for ischemia studies in cultured neural cells [28, 30, 31]. Cultures were first washed twice with ischemic medium and then incubated with 0.8 ml ischemic medium per 35 mm culture dish for 0, 0.25, 2, 4 and 6 h.

Post-ischemia studies were carried out on astrocytic cultures that had been subjected to 3 h of ischemic treatments. Ischemic medium was replaced by freshly prepared serum-free DMEM. Cultures were transferred to a standard normoxic CO_2 incubator for post-ischemic treatment of 1 or 3 h [32].

MK-801 and CNQX Treatments

Astrocytes can express α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA-R). Selective antagonists of NMDA-R and AMPA-R were adopted: MK-801, a selective non-competitive NMDA-R antagonist, and CNQX, a selective competitive AMPA-R antagonist. Cultures were pretreated with different dosages of MK-801 (0, 1, 5, 10, 20 μM) (Sigma–Aldrich, MO, USA) or CNQX (0, 10, 20 40, 60 μM) (Sigma–Aldrich, MO, USA) for 15 min. Astrocytic cultures were then incubated in MK-801 or CNQX containing ischemic medium at 37°C for 0, 0.25, 2, 4 and 6 h in the ischemic chamber.

NOS1 Measurement

Proteins of ischemia-treated astrocytes were extracted according to Chen et al. [24]. Briefly, astrocytes were placed on ice, washed twice with chilled PBS, and protein was collected. Cell suspensions were homogenized using an ultrasonic processor (Ultrasonic and Materials Inc., CT, USA). The cell lysate was centrifuged at 13,000 rpm for 10 min at 4°C (Biofuge 15R; Heraeus Sepatech, Germany). The protein content of the supernatant was determined by Lowry's method and stored at −70°C until use.

Standard Western blot procedure was followed for NOS1 measurement. Samples were separated on a 10% SDS–polyacrylamide gel. Proteins were transferred onto a PVDF membrane (Millipore, MA, USA). NOS1 was detected by NOS1 polyclonal antibody (1:1000) (Santa Cruz Biotechnology, Inc., CA, USA), anti-rabbit IgG horse-radish peroxidase conjugated antibody (1:1500) (Beijing Zhongshan Biotechnology Co., China), and an enhanced chemiluminescent (ECL) Western blot detection kit (Santa Cruz Biotechnology Inc., CA, USA). Actin was detected by actin monoclonal antibody (Sigma–Aldrich, MO, USA) and served as an internal control to quantify equal loadings.

Statistical Analysis

Results were analyzed by One-way ANOVA with Bonferroni post-test or Student's *t*-test using PRISM®4.0 (GraphPad Software, Inc, CA, USA) as mean ± S.E.M. Results were considered significant at $P < 0.05$.

Results

Measurements of NMDA-R Subunits in Primary Cultures of Astrocytes

GFAP and MAP2 are markers specific for astrocytes and neurons, respectively. Over 95% of cells in the primary culture of astrocytes were GFAP positive by immunofluorescent staining, indicating the high purity of the cultures. The absence of MAP2 positive cells indicated that the cultures are lack of neuronal components (Fig. 1).

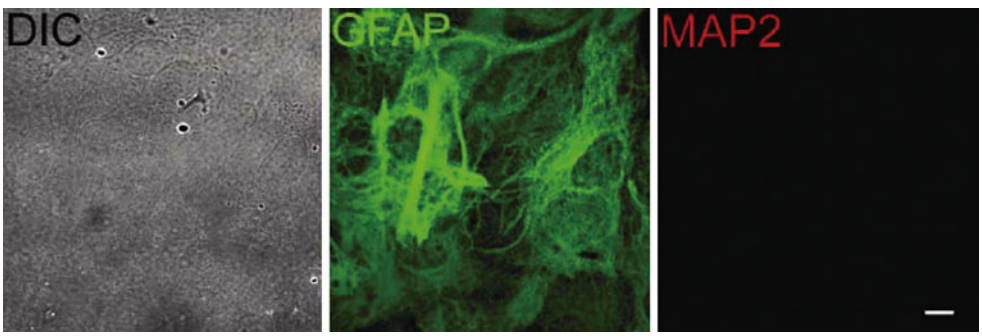

Fig. 1 Purity identification in primary cultures of astrocytes prepared from cerebral cortex. Astrocytes in 4 week-old culture were double stained with astrocytic specific marker, GFAP, and neuronal specific marker, MAP2. Cultures were then imaged by differential interference contrast (DIC) and confocal microscopy. No MAP2-positive cells were detected indicating the culture did not have any neuronal component. *Bar* = 10 μm

The mRNA levels of NMDA-R subunit in 3-week-old primary cultures of cortical astrocytes and in 1-week-old primary cultures of cortical neurons were measured by RT-PCR (Fig. 2a). The neuronal samples showed strong bands for the NMDA-R subunit NR1, NR2A and NR2B. These bands were also found in the astrocytic samples, indicating that astrocytes are also capable to express these NMDA-R subunits, though their levels were lower than neurons for all three subunits.

The mRNA levels of NMDA-R subunits were measured in 1- to 4-week-old primary cultures of astrocytes by RT-PCR (Fig. 2b–d). NR1 mRNA was significantly down-regulated to about 50% of 1-week old culture in 2-week-old cultures (Fig. 2b). The NR1 mRNA remained at this low level in 3- and 4-week-old astrocytes. In contrast, NR2A mRNA levels were significantly up-regulated by more than 60% in 2-week-old cultures and continued to increase in 3- and 4-week-old culture, reaching a level 250% higher than those in 1-week-old cultures (Fig. 2c). NR2B mRNA was also significantly up-regulated in 2-week-old cultures by about 30%. It remained at this level in 3- and 4-week-old cultures with a slight and non-significant decrease (Fig. 2d). To confirm whether these mRNAs were translated into their proteins in astrocytes, we

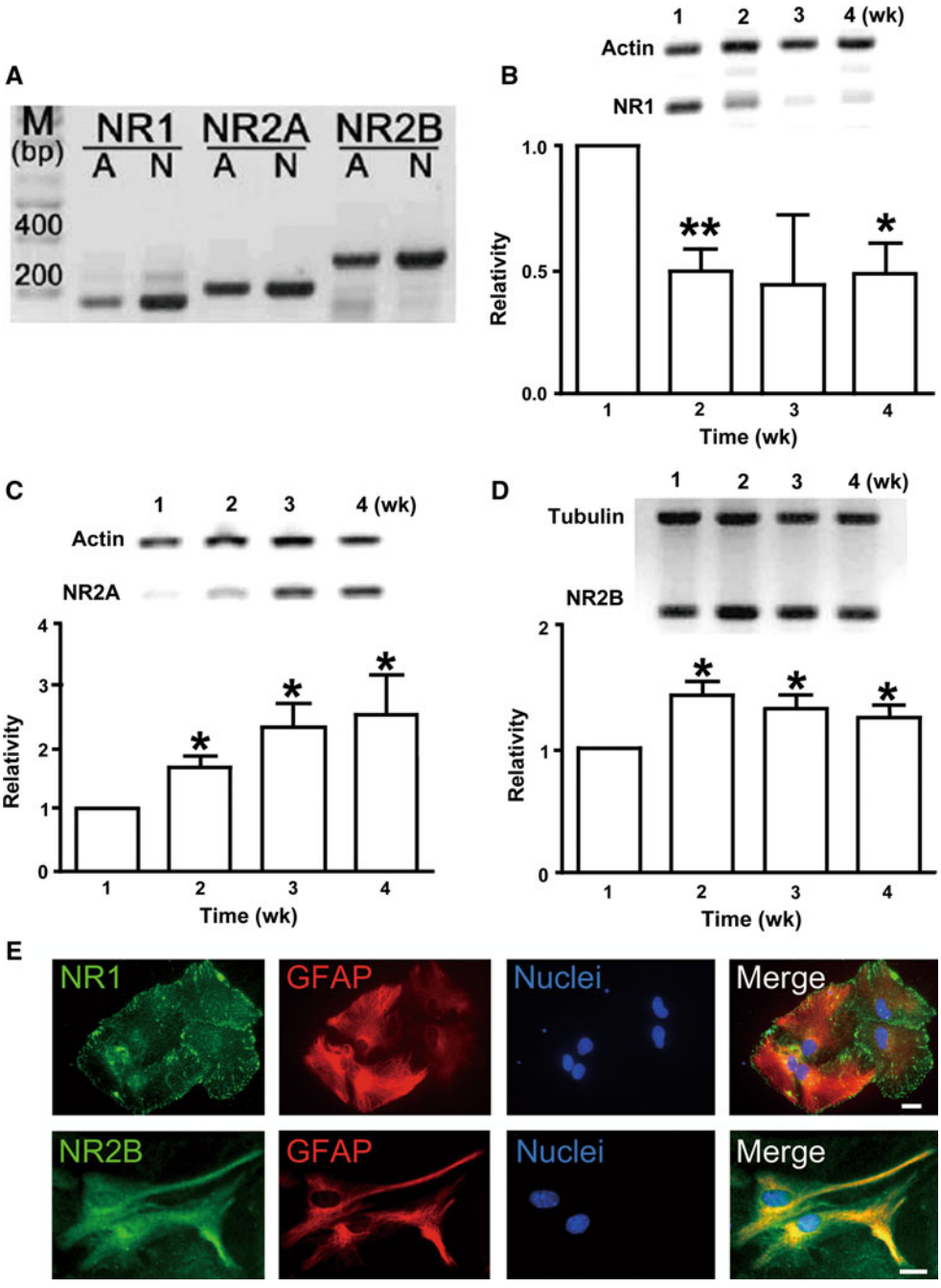

Fig. 2 Measurement of NMDA-R subunit mRNA in primary cultures of astrocytes. **a** The mRNA levels of three NMDA-R subunits, NR1, NR2A and NR2B, were examined in primary cultures of astrocytes. Neuronal samples were used as positive control. All three subunits could be detected in astrocytes. *A* astrocyte, *N* neuron. **b** The mRNA level of NR1 decreased significantly (~50% of week-1 levels) in astrocytes during the 4 weeks of development in primary cultures. (n = 5). **c** The mRNA level of NR2A increased significantly (~200% of week-1 levels) in astrocytes during the 4 weeks of development in primary cultures. (n = 5). **d** The mRNA level of NR2B peaked at 2 weeks (~130% of week-1 levels) in astrocytes and gradually decreased in the subsequent weeks in primary cultures. (n = 5). **e** Immunofluorescent staining of NR1 and NR2B in 1-week-old astrocytes in cultures. NR1 was detected in the cell membrane and NR2B was mainly in the cytoplasm. *Bar* = 10 μm

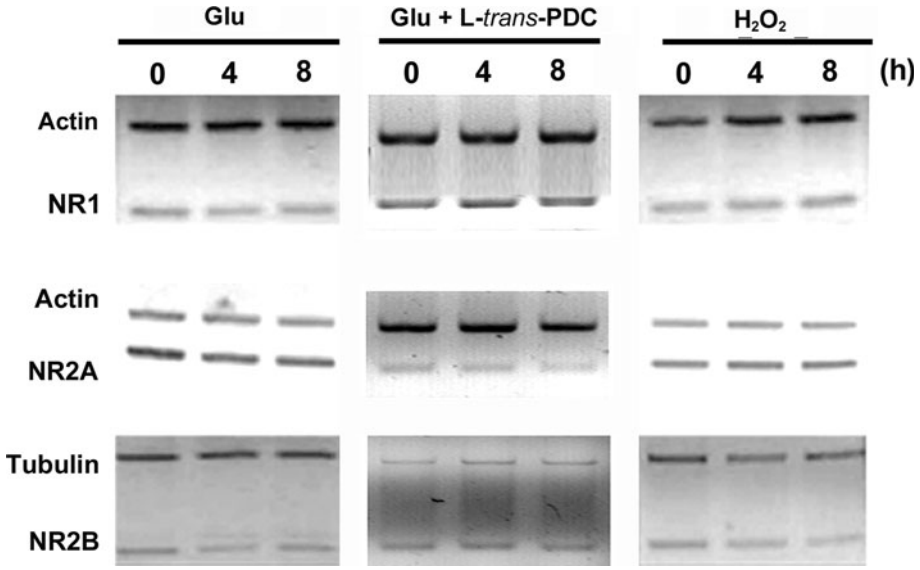

Fig. 3 Effect of Glu and H_2O_2 on the levels of NMDA-R subunit mRNA. Astrocytes were treated with glutamate (Glu, 1 mM), glutamate and L-*trans*-PDC (50 μM) or H_2O_2 (200 μM) for 0, 4, and 8 h. The levels of NR1, NR2A and NR2B mRNA did not show any obvious change

immunostained 1-week-old cultures using antibodies against NR1 and NR2B. We were able to identify some NR1 and NR2B positive astrocytes in the culture (Fig. 2e), indicating mRNA translation. The large numbers of NR1 and NR2B negative astrocytes indicated low protein expression of these subunits in 1-week-old culture. This may also reflect the heterogeneity of astrocytes in the expressions of these NMDA-R subunits.

Effects of Glutamate or H_2O_2 Treatment on NMDA-R Subunit mRNA Levels

Glutamate and H_2O_2 are known to induce changes in neuronal NMDA-R subunit expression [33, 34]. To investigate whether similar effect was exerted on astrocytes, we treated astrocytes with glutamate or H_2O_2. The mRNA levels of NR1, NR2A and NR2B remained unchanged during the 8 h of glutamate or H_2O_2 treatment (Fig. 3). We also treated astrocytes with glutamate transporter inhibitor, L-*trans*-PDC, under glutamate treatment. This will allow us to clarify whether the lack of glutamate effect on these NMDA-R subunit mRNAs was due to the drastic changes in extracellular and intracellular glutamate concentrations caused by the high affinity uptake of the glutamate by its transporter. There was also no significant difference in the NR1, NR2A or NR2B mRNA levels during glutamate treatments in the presence of L-*trans*-PDC (Fig. 3). Therefore, unlike neurons, astrocytes did not show detectable transcriptional responses to glutamate or H_2O_2 treatment with respect to these three NMDA-R subunits.

Effects of Ischemia and Post-Ischemia on NMDA-R Subunit mRNA Levels

Ischemia and post-ischemia could selectively induce changes in NMDA-R subunit mRNA levels in 4-week-old

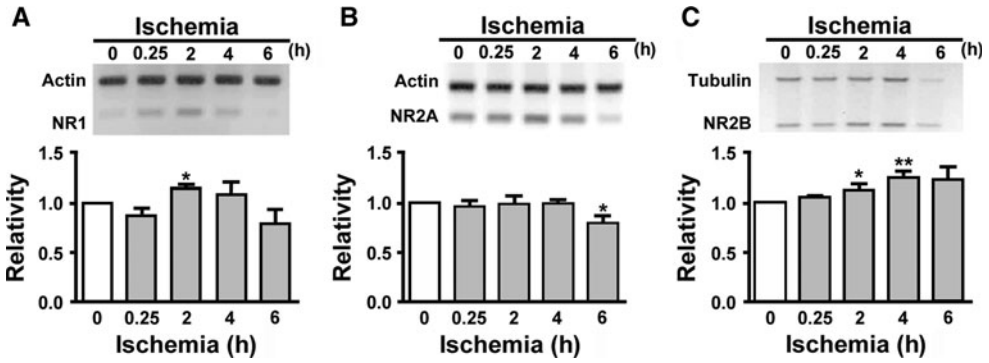

Fig. 4 Effect of ischemia on the levels of NMDA-R subunit mRNA in astrocytes. Primary cultures of astrocytes were subjected to ischemia treatment. The mRNA levels of NMDA-R subunits were measured by RT-PCR (n = 5). **a** Ischemia induced a significant up-regulation of NR1 mRNA at 2 h (~120%). **b** Ischemia did not induce any up-regulation of NR2A mRNA, but a significant down-regulation at 6 h of ischemia. **c** Ischemia induced a gradual increase in the NR2B mRNA expression in the 6 h of ischemic treatment, and the increment (~20%) was already becoming significant at 2 h of ischemia

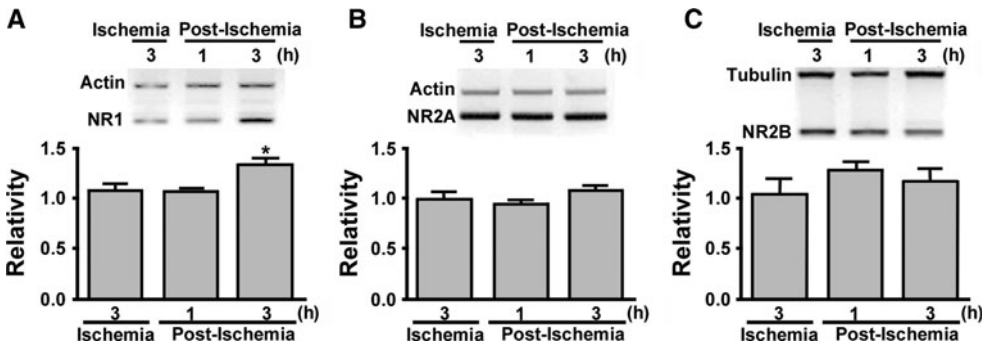

Fig. 5 Effect of post-ischemia on the levels of NMDA-R subunit mRNA in astrocytes. Primary cultures of astrocytes were subjected to 3 h ischemia before incubating in a normoxic incubator for 1 or 3 h of post-ischemia treatments. **a**. The mRNA level of NR1 was significantly increased (~130%) at 3 h of post-ischemia. **b**, **c** There was no statistically significant change detected in the mRNA levels of NR2A and NR2B during post-ischemia treatment (n = 3)

astrocytic cultures. During ischemia treatment, NR1 mRNA level was initially up-regulated by about 20% at 2 h of ischemia, but fell to a level lower than the control at 6 h (Fig. 4a). NR2A mRNA level remained stable throughout the first 4 h of ischemia, but had significantly decreased by 15–25% at 6 h (Fig. 4b). NR2B mRNA level was significantly increased by 15–20% at 2 h of ischemia and further increased to 25% higher than the control by 4 h of ischemia treatment (Fig. 4c).

After 3 h of ischemia treatment, cultures were incubated for 1 or 3 h under normal incubation condition—post-ischemia. By 3 h post-ischemia, the level of NR1 mRNA had significantly increased by about 30% (Fig. 5a). However, NR2A and NR2B mRNA levels did not undergo significant change although a rise in NR2A but a fall in NR2B mRNA levels were noted from 1 to 3 h post-ischemia (Figs. 4b, 5c). Thus, distinct responses to ischemia and post-ischemia treatments with regard to transcriptional changes in NMDA-R subunits appeared to exist in astrocytes in culture.

NOS1 Protein

The spatial, temporal and inducible changes in NMDA-R mRNA and the low levels of their proteins detected by immunostaining suggested that astrocytes process low level of these subunit proteins. Whether these NMDA-Rs are functional was therefore examined. NOS1 is downstream of NMDA-R signaling cascades. NMDA-R activation increases NOS1 protein levels, leading to the release of NO [9, 35]. NOS1 levels could thus be used as a functional assay for NMDA-R activity.

NOS1 protein levels in the 4-week-old control cultures of astrocytes were low as measured by Western blot. Interestingly, NOS1 protein levels had significantly increased at 2 h and peaked at 4 h of ischemia (Fig. 6a). During the first hour post-ischemia, the NOS1 protein level

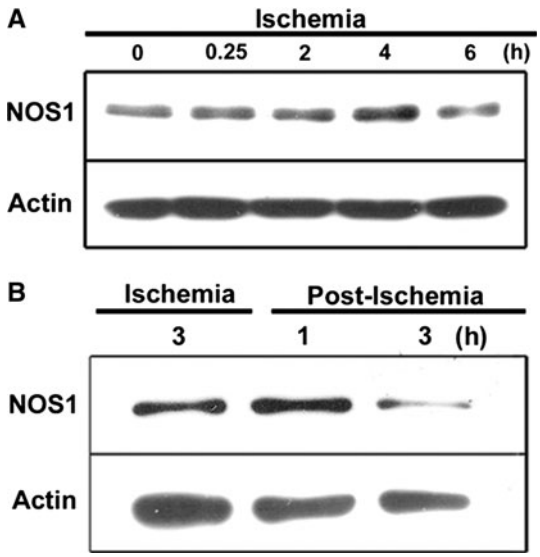

Fig. 6 Effect of ischemia and post-ischemia on the level of NOS1 protein. **a** Ischemia induced a significant up-regulation of NOS1 protein from 2 h and peaked at 4 h. **b** During the first hour of post-ischemia, the NOS1 protein level remained at a similar level as at the 3 h ischemic incubation. A significant decrease in NOS1 level was measured in astrocytes at 3 h of post-ischemia treatment

was maintained at a level as high as at 3 h of ischemia, then it decreased at 3 h of post-ischemia (Fig. 6b).

Astrocytes can express AMPA receptors, which are closely linked with NMDA-R in many functional aspects in neurons [36, 37]. We treated cultures of astrocytes with the AMPA-R specific inhibitor, CNQX, to determine whether AMPA-R plays a role in the elevation of NOS1 protein in astrocytes under 4 h of ischemia. We found that 20 μM CNQX did not prevent the increase of NOS1 protein at 4 h of ischemia (Fig. 7a), neither in various dosages treatments up to 60 μM (Fig. 7b).

To elucidate whether the ischemia-induced increase in NOS1 protein levels in astrocytes was caused by the presence of functional NMDA-R, we treated astrocytes

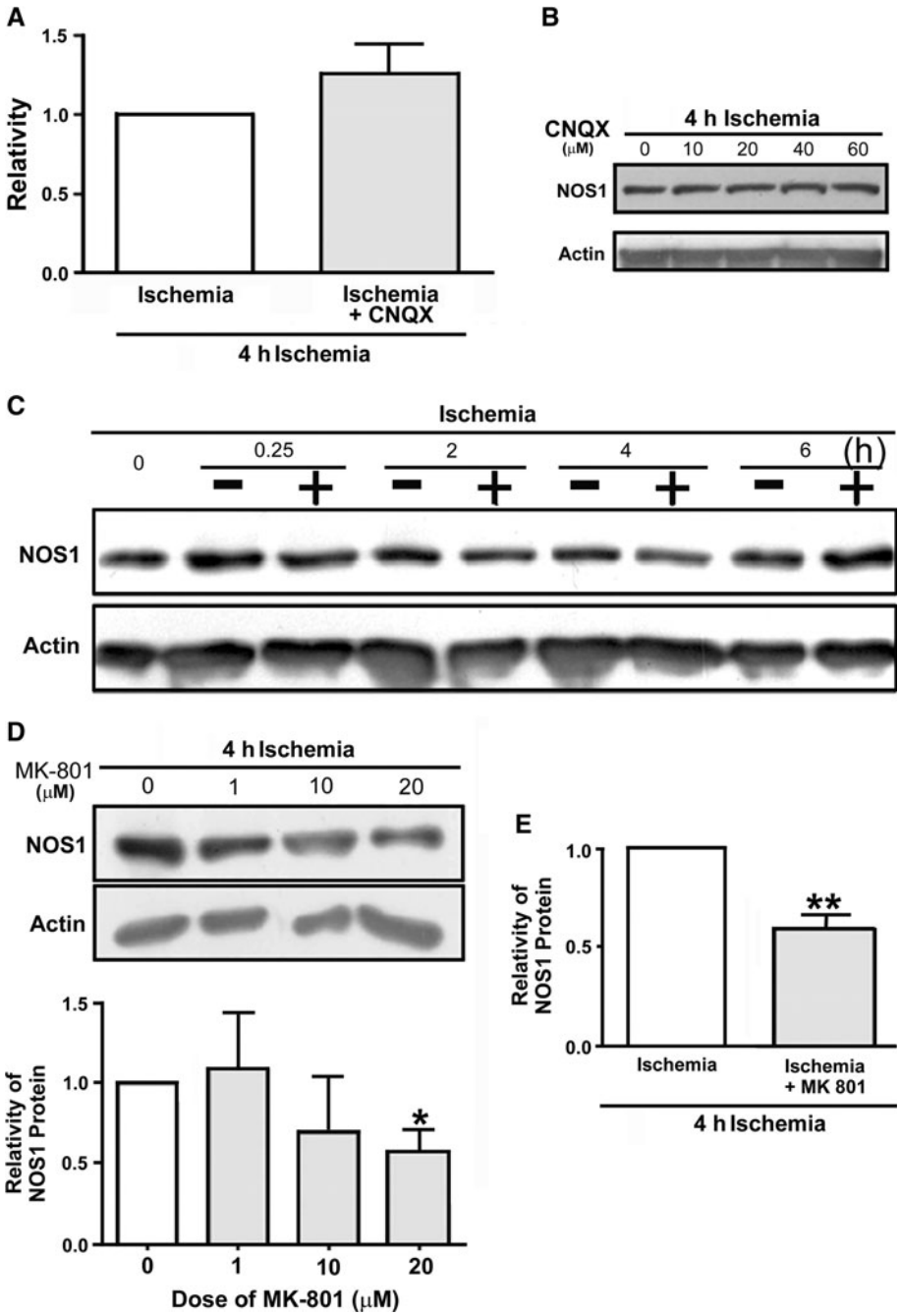

Fig. 7 Effect of CNQX and MK-801 on NOS1 protein in astrocytes under ischemia treatment. **a** Astrocytes in cultures were pre-treated with CNQX before the 4 h of ischemia treatment. Control was astrocytes without CNQX treatment. No significant changes in NOS1 level was observed between the control and CNQX treated astrocytes. (n = 10). **b** Different dosages of CNQX were applied to astrocytes subjected to 4 h of ischemia treatment. The NOS1 level was not affected by any of the dosages of CNQX used. **c** Astrocytes were pre-treated with MK-801 before ischemia treatment. Control was astrocytes without MK-801 treatment. The ischemia-induced up-regulation of NOS1 was inhibited at 0.25, 2, 4 h and became significant at 4 h. **d** The inhibitory effect of MK-801 on NOS1 was dose-dependent. The inhibition became significant at 20 μM. (n = 3). **e** The effect of 20 μM MK-801 on NOS1 expression with 4 h ischemia treatment (n = 6)

with MK-801, a selective antagonist of NMDA-R. A 20 μM MK-801 treatment resulted in more than 40% reduction in NOS1 protein levels with the inhibitory effect being observed already at 0.25, 2, and 4 h of ischemia. The inhibitory effect was weakened at 6 h of ischemia (Fig. 7c). Treatment with increasing dosages of MK-801 appeared to have confirmed the inhibitory effect of MK-801 on NOS1 protein levels during ischemia (Fig. 7d). This inhibitory effect was statistically significant at 20 μM (Fig. 7d, e). This confirmed the presence of inducible and functional NMDA-R in astrocytes.

Discussion

Since the identification of NMDA-R 30 years ago, it has been shown that the receptor is involved in many neural functions, such as synaptic transmission, synaptic plasticity, development and neurodegeneration [3–5]. Abel Lajtha and his group recently have reported the deficits in NMDA-R mediated neurotransmission may underlie dopaminergic hyperactivity in schizophrenia [38]. However, studies on NMDA-R were mainly limited to neurons. There are only a few experimental evidences which revealed the effect of

NMDA-R activity on astrocytes. For instance, studies have demonstrated that activation of neuronal NMDA-R could affect GFAP expression, glutamine synthetase activity and the intracellular calcium level of astrocytes in vivo [15, 16, 39].

Once considered as glue, astrocytes have continued to reveal their surprising functional activities: sensing and regulating tri-partite synaptic activities as well as transporting, releasing and metabolizing glutamate, the most abundant excitatory transmitter and the internal ligand of NMDA-R [14, 25]. However, whether NMDA-R expressed in astrocytes themselves has been controversial for a long time. Although at lower levels than in neurons, the identification of the mRNA of the three NMDA-R subunits, NR1, NR2A and NR2B, in primary cultures of astrocyte was an encouraging indication of the presence of this receptor in astrocytes. Our immunostaining results demonstrated some astrocytes in cultures could express NR1 and NR2A. The NR2B subunit was difficult to detect in astrocytes by immunostaining, indicating the basal level of this NMDA-R subunit in astrocytes was very low. Nonetheless, our findings regarding the significant up-regulatory effects of ischemia on NR1 gene expression and the functional NMDA-R activity as determined by the specific MK-801 inhibition on the ischemia-induced elevation of NOS1 levels confirmed the existence of inducible and functional NMDA-R in astrocytes.

Glutamate and H_2O_2 are known to be closely associated with NMDA-R function in neurons under normal conditions and during brain injuries such as ischemia, in which they both stimulate gene expression and regulate activity of NMDA-R. However, they did not exert detectable effect on the levels of NMDA-R subunits in astrocyte, indicating a difference in astrocytic and neuronal responses to these external factors in regard to NMDA-R expression. Glutamate is the internal ligand of NMDA-R and a regulator of neuronal NMDA-R gene expression. The lack of detectable glutamate effect on astrocytes indicated that NMDA-R activation and induction pathways in astrocytes may differ from those in neurons.

We found that the transcript level of the essential subunit of NMDA-R, NR1, gradually decreased during development to a very low level, which was maintained but at a level difficult to be detected in 2–4 week-old cultures of astrocyte. In contrast, NR1 mRNA increases in neurons after birth both in vivo and in vitro [40, 41]. Thus the NR1 expression profile is different in neurons and astrocytes. This decrease in NR1 mRNA in astrocyte during maturation in culture might be one explanation for the previous reports on the lack of functional NMDA-R expression in astrocyte [42].

The rapid up-regulation of NR1 mRNA after 2 h of ischemia demonstrated that external factors may play an important role in NR1 expression in astrocytes. This may be another explanation for the controversy in reporting over the existence of functional NMDA-R in astrocytes. The lower NR1 mRNA level observed in mature astrocytes combined with a lack of external inducing factors (e.g. ischemia or interaction with neurons) might confine and conceal astrocytic NMDA-R activity below a detectable threshold.

The up-regulation of NR2A and NR2B levels in astrocytes as a function of age in culture was similar to that reported in neurons [40, 43]. Functional NMDA-R requires NR1. Thus from observations of astrocyte maturation in culture, the down-regulation of NR1 implies a shortage of this subunit and the up-regulation of NR2A or B implies storage in astrocyte cytoplasmic pool. To date, only NR1 has been reported as potentially able to exist in a cytoplasmic pool by virtue of an ER retention signal [44–46]; although trafficking of other NMDA receptor subunits among subcellular compartments has also been demonstrated [47, 48]. Therefore, we hypothesize that NR2 subunits are retained in their compartments until required [49]. However, we cannot rule out the fact that no detectable increase in NR2A or NR2B subunit mRNA in concordance to the up-regulation of NR1 expression might mean that other NR2 or NR3 subunits such as NR2C, NR2D, NR3A or NR3B that we have not studied being induced instead.

The ratio of NR2A to NR2B mRNA appeared to vary during development (Fig. 2). Switching of subunits from NR2B to NR2A occurred in granule cell maturation in the cerebellum [50]. Neonatal brain NMDA-Rs are also rich in NR2B, which is dramatically reduced in proportion to NR2A as the brain develops [51]. This appears to be similar to the trend we observed in developing astrocytes in culture [52].

Variation in neuronal NMDA-R subunit levels has also been observed in various pathological conditions in vivo, especially during ischemia and post-ischemia [50, 53, 54]. The two modifying subunits, NR2A and NR2B, influence channel properties of NMDA-R [43, 55]. A NR2A to NR2B shift in hippocampal neurons exposed to ethanol makes NMDA-R more sensitive to glutamate [56] and prolongs its opening time due to the lack of the Ca^{2+} dependent inactivation [57], thus allowing a prolonged Ca^{2+} influx with severe consequences [58]. Other studies also reported the switching of NR2B to NR2A after ischemia, a change which might protect the neuron [59]. Our results from astrocytes in culture also indicated a reduction in NR2B and an increase in NR2A from 1 to 3 h post-ischemia, albeit non-significant.

The up-regulation of NR2B mRNA during ischemia is consistent with previous findings in astrocytes in anoxic hippocampal co-cultures. However, these NMDA-R subunits were not detected in pure cultures of hippocampal

astrocyte under anoxia, [23]. Differences between culture systems, methods of injury and a short detection time course may have partly contributed to this apparent divergence, along with the reported heterogeneity of receptor expression among astrocytes and the difficulties in detecting low levels of protein by immunohistochemistry. Such observations of alterations in NR2 subunits in astrocytes are consistent with some of the reported observations in neurons, and are likely to affect brain function and participate in molecular mechanisms of resistance or susceptibility to injury [60].

NOS1 expression in neuronal cultures can be modulated by NMDA-R activity and is blocked by the specific NMDA-R antagonist, MK-801 [61]. Thus expression changes of NOS1 can reflect the activation of functional NMDA-R [9, 10, 62]. NOS1 expression has been observed in cultured astrocytes [63], but ischemia-induced NOS1 up-regulation in astrocytes has not been reported. The dose-dependent inhibitory effect of MK-801 on ischemia-induced NOS1 up-regulation confirmed that astrocytes possess functional NMDA-R and demonstrated that the increase of NOS1 protein in astrocytes under ischemia was attributable to activation of NMDA-R in astrocytes. This is in consistency with the observed up-regulation of functional subunit NR1 mRNA in astrocytes under ischemia treatment. NOS1 in neurons is found to also link to another glutamate ionotropic receptor, AMPA-R which is also known to facilitate NMDA-R activation [64]. Astrocytes have been shown to express functional AMPA-R [37]. Our data on the inhibition of AMPA-R with CNQX did not show any detectable effect on NOS1 protein expression. Therefore, AMPA-R was considered not to involve in the induction of NOS1 protein in astrocytes during ischemia.

We noticed that during 3 h of post-ischemia, the NOS1 protein gradually returned to the basal level while mRNA levels of the functional NR1 subunit continued to increase, suggesting a continued requirement for NMDA-R. A decrease in NOS1 protein has been reported in cerebral cortical neuron cultures treated with post-hypoxia, and this change was considered to be mediated by NMDA-R through alterations of subunits NR2A and NR2B [61]. The trend towards an increased NR2A/NR2B ratio after ischemia may reduce glutamate sensitivity [58], thus to reduce NOS1 protein levels and its activity [65]. The decrease of NOS1 in astrocytes under post-ischemia treatment was consistent with results from neuron and serve as further evidence for the presence of functional NMDA-R in astrocytes.

This study has demonstrated that astrocytes process the capacity in expressing NMDA-R subunit mRNA and proteins even in the absence of neurons. The associated ischemia-induced NR1 and NOS1 expression and the inhibitory effect of MK-801 verified the presence of functional NMDA-R in cultured astrocytes. These findings confirmed the intrinsic capability of astrocytes to express NMDA-R under both physiological and pathological conditions, and brought to light the potential involvement of specific NMDA-R functions in astrocytes under ischemia, and other pathological and physiological conditions.

Acknowledgments This work was supported by the National Natural Science Foundation of China (30270426, 30470543, 30670644, 30870818, 31070974), the Beijing Natural Science Foundation (7032026, 7051004, 7091004), the National High Technology Research and Development Program of China (863 Program, 2006AA02Z452) and the National Basic Research Program of China (973 Program, 2011CB504400).

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